Enhanced botulinal toxin development in beef sausages containing decolourized red blood cell fractions

MILLER, A.J., MURPHY, C.A., CALL, J.E., KONIECZNY, P. & UCHMAN, W. 1993. Enhanced botulinal toxin development in beef sausages containing decolourized red blood cell fractions. *Letters in Applied Microbiology* **16**, 80–83.

Toxin production by *Clostridium botulinum* was studied in a model cured beef sausage containing decolourized dried bovine red blood cells (RBC), including intact RBC, acetone-treated RBC, enzyme-treated RBC, peroxide-treated RCB or plasma. Samples were formulated with beef shoulder, curing agents and spores of proteolytic strains of *Clostridium botulinum*. Vacuum packaged samples were heated to 72°C, stored at 28°C, and tested weekly. Sausages contained iron levels proportional to the iron in the blood fraction. Residual nitrite levels varied between <10–40 μg g^{-1}. Toxin was detected earlier in samples containing higher levels of iron except for acetone-treated RBC. Higher pH values were associated with shorter times to toxin detection. We conclude that the RBC decolourization method can significantly modulate *Cl. botulinum* growth and toxigenesis.

Blood fractions are potentially useful ingredients to increase protein functionality (Tybor *et al.* 1975). However, haeme reduces acceptability because it is dark in colour and imparts a characteristic flavour. Decolourization by haeme reduction has been used to overcome these shortcomings, and methods include: solvent extraction, oxidation, hydrolysis, adsorption and bleaching (Uchman and Konieczny 1988). Microbiological safety questions regarding blood use in cured meat products focus on the risk of human botulism (Lücke 1985; Simunovic *et al.* 1985). Extracellular iron was hypothesized to inactivate nitrite, which inhibits toxin production (Tompkin *et al.* 1979). Cured sausages treated with iron-containing blood fractions produced *Cl. botulinum* neurotoxin earlier than control sausages or those containing various blood products having low iron levels (Miller and Menichillo 1991). In the present study we compared cured sausages containing dried red blood cells (RBC) with those containing decolourized RBC or plasma for their effect on botulogenesis.

Materials and Methods

BLOOD FRACTIONS

Dried RBC fractions were prepared according to Uchman and Konieczny (1988, 1989). Briefly, RBC fractions were prepared from fresh bovine blood, centrifuged, then freeze dried to obtain the cellular fraction, which was used as the reference sample (RBC). Peroxide decolourization (PER) was accomplished by RBC haemolysis with distilled water (1 : 9), heat treatment (70°C for 15 min), cooling to 20°C, and treatment with 5% H_2O_2 (1 : 1, v/v). Preparations were centrifuged at 3000 *g* for 10 min, then the sediment was washed with distilled water and centrifuged again. The sediment was lyophilized. The acetone RBC (ACE) fraction was prepared by haemolysis of RBC by a freeze-thaw cycle, followed by heating at 90°C for 15 min, then cooling at 20°C. The preparation was then extracted with acetone until there was a colourless eluent. The light-beige residue was washed with distilled water, centrifuged at 3000 *g* for 10 min, then dried in 20°C air. The enzyme-treated sample (ENZ) was prepared by haemolysis of the RBC fraction with distilled water (1 : 2), pH adjustment to 4·0 (using 1 mol l^{-1} HCl), followed by enzymatic hydrolysis. Conditions for hydrolysis were 45°C for 18 h using Proteopol BP-S (Wytwornia Preparation Enzymatycznych; Jasto, K/Krosno, Poland) with activity of 65000 haemoglobin units (10 ml enzyme solution/100 g of RBC). Proteopol BP-S is obtained from *Bacillus subtilis* and contains a complex of proteolytic and some amylolytic enzymes. The hydrolysate was centrifuged as above and supernatant fluid lyophilized. Dried bovine plasma (PLS) was purchased from the American Protein Corporation (Ames, IA).

STRAINS AND SPORE PREPARATION

Twenty strains of *Cl. botulinum* spores were used, including 11 type A (2-OPLALCA, 174091A, 3, 4, 33A, 25763, 62A, 69, 78, 426, 429) and nine proteolytic type B (169, 383, 624, 999, 8688R, 5, 770B, 53B, 7949) strains. Each strain was prepared from cultures of heat-shocked (80°C for 10 min) stock spores in botulinal assay medium (BAM) as described previously (Miller and Menichillo 1991). A spore mix was prepared from equal numbers of the 20 strains giving a total of $2{\cdot}1 \times 10^5$ spores per ml.

PRODUCT FORMULATION

Model cured beef sausages were formulated with beef chuck, 10% water, 3% dried bovine blood fractions, 2·5% NaCl, 550 $\mu g\ g^{-1}$ sodium ascorbate and 156 $\mu g\ g^{-1}$ $NaNO_2$. Blood fractions included: RBC, ACE, ENZ, PER and PLS. Two controls were formulated without blood fractions and either 156 $\mu g\ g^{-1}$ $NaNO_2$ or without nitrite. All food ingredients were homogenized for 2 min in a food processor to yield about 600 g batch^{-1}. Formulations were then inoculated with a target level of 300 spores g^{-1} and thoroughly mixed. Ten gram ($\pm 0{\cdot}1$ g) aliquots were vacuum sealed in low oxygen-permeable film (IKD All-Val #13, O_2 permeation = 1·0 cc/100 in^2/24 h at 25°C, International Kenfield Distributing Co., Rosemont, IL); these were heated at 72°C for 20 min, cooled and stored at 28°C.

SAMPLING AND ANALYSIS

Packages were removed weekly and samples prepared by adding sterile distilled water and blending in a Stomacher (A.J. Seward, London) and centrifuged at 3000 *g* for 5 min. The pH was determined on the liquid homogenates using a combination electrode attached to a Radiometer PHM82 meter (Radiometer, Copenhagen, Denmark). Nitrite levels were estimated by the AOAC (1984) procedure. Botulinal toxin was determined using the mouse bioassay (Miller and Menichillo 1991). Total iron levels in meat samples and blood fractions were determined using AOAC (1984) ashing and atomic absorption spectroscopy procedures. Viable spore counts were estimated by a 3-tube MPN. Sample weights for MPN analysis were 0·1, 0·01, 0·001 g and were suspended in TPGY broth and incubated anaerobically at 35°C for 7 d. All results were statistically analysed using RS/1 (BBN, Cambridge, MA), a VMS-based software program used to store and analyse technical data.

Results

Total iron levels ($\mu g\ g^{-1}$) in blood fractions were RBC = 1935, ACE = 1496, PER = 1406, ENZ = 130, and PLS = 63. Acetone or peroxide decolourization reduced iron levels only by 23% and 27%, respectively, compared to the reference RBC sample, while enzymatic hydrolysis reduced iron levels by 93%. Beef contained approximately 20 μg iron g^{-1} meat. Total iron levels ($\mu g\ g^{-1}$) in the model beef sausages were RBC = 108, ACE = 70, PER = 55, ENZ = 21, PLS = 17, and control, with no added blood fraction (NoBL) = 17. Product iron levels increased directly with the iron content of the blood fraction. Control and plasma-containing sausages had marginally less iron than raw beef because of the dilution by curing agents and water. Residual nitrite levels immediately after cooking were 10–40 $\mu g\ g^{-1}$; within 1 week all values were <10 $\mu g\ g^{-1}$. Ingoing spore levels averaged 288/g for the two trials.

Differences were observed in pH values among the various model sausage treatments (Fig. 1), with a significant association ($P < 0{\cdot}01$) between final pH values and time to toxicity. Generally, higher pH values were associated with shorter times to toxin detection. PER and RBC containing samples were toxic at 1 week and had pH values at that time of 6·21 and 6·27, respectively. PLS containing samples had a pH value of 5·89 at the time of toxicity (3 weeks). Controls, having nitrite and no blood fraction (NoBL), ACE and ENZ samples had pH values of 6·03, 5·86 and 5·83, respectively at the time of toxicity (2 weeks). Samples toxic at 1 week showed a marked tendency to become more alkaline with longer incubation time. For example, the pH of PER-treated sausages increased from 5·98 to 6·68, and the pH of RBC

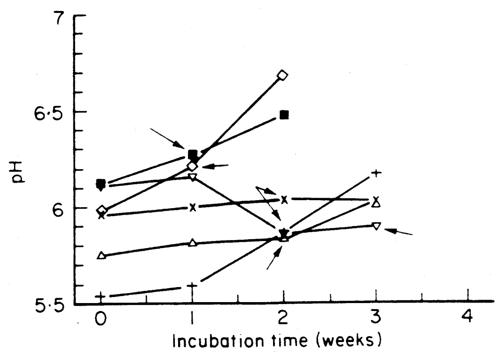

Fig. 1. Effect of incubation time on model sausage pH. ■, RBC (red blood cells); +, ACE (acetone-extracted RBC); ▽, PLS (plasma); ◇, PER (peroxide-treated RBC); △, ENZ (enzyme-treated RBC); ×, NoBL (control, containing 156 μg g^{-1} $NaNO_2$ and no blood fractions). Arrows indicate time at which first significant sign of toxicity occurred.

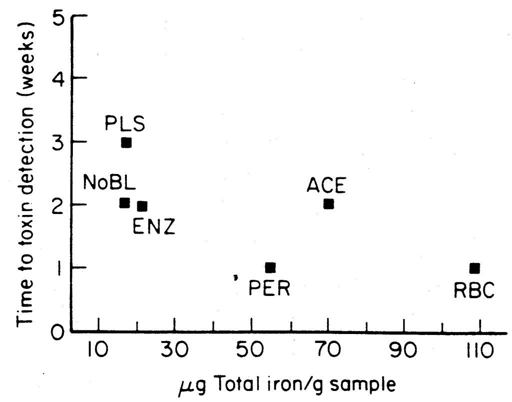

Fig. 2. Relationship between iron levels and *Clostridium botulinum* toxin development in a model beef sausage.

containing samples increased from 6·12 to 6·47 over a 2 week test period. The PLS sample (toxic at 3 weeks), conversely, decreased slightly from pH 6·1 to 5·9. The pH trend of the samples which were toxic at 2 weeks was inconsistent. The control (NoBL) showed little change in pH (5·97 to 6·02) over 3 weeks. Sausages containing ACE exhibited a 0·7 pH increase (5·54 to 6·17). The pH values of sausages with ENZ increased from 5·76 to 6·02 over this period.

Toxin data are shown in Table 1. Control samples (no nitrite, no added blood) were toxic at 1 week. When nitrite, but no blood, was added, the time to toxin detection was extended to 2 weeks. RBC samples became toxic at 1 week, as was PER. Products which had incorporated ENZ and ACE exhibited neurotoxin at 2 weeks. PLS containing samples showed toxin by 3 weeks. Generally, toxin was detected earlier in samples containing higher levels of iron (Fig. 2). PLS samples had the lowest iron level and longest time before toxin was observed. In contrast to PLS, RBC had the highest iron level and shortest toxin lag. Control samples (nitrite, no blood) and ENZ-treated sausage samples had similar iron levels and toxin development patterns (2 weeks). A protective effect of plasma is indicated by the longer toxin lag period, despite the similarities in iron levels of the PLS, NoBL control and ENZ-treated sausages. ACE (70 μg iron g^{-1}) and PER (55 μg iron g^{-1}) treated samples had approximately 50% lower iron levels than RBC and they became toxic at 2 and 1 week, respectively.

Discussion

The principal finding of the present investigation is that addition of certain decolourized blood fractions to processed meat may increase risk for the development of botulinal toxin. This supports previous results from this laboratory

Table 1. Time to toxin detection in model cured beef sausages*

Weeks at 28 C	3% Blood fraction addition					Control (no blood)	
	RBC	ENZ	ACE	PER	PLS	$+NO_2$	$-NO_2$
0	0/6†	0/6	0/6	0/6	0/6	0/6	0/6
1	6/6	0/6	0/6	5/6	0/6	0/6	6/6
2	5/6	5/6	6/6	6/6	0/6	6/6	6/6
3		6/6	6/6		5/6	6/6	
4					6/6		

* Results are pooled data from duplicate trials.
† Number of packages having toxin/total number of packages.
Abbreviations: RBC, red blood cells; ENZ, enzyme-treated RBC; ACE, acetone-extracted RBC; PER, peroxide-treated RBC; PLS, plasma; NO_2, sodium nitrite.

which demonstrated that added iron from intact bovine blood fractions decreased the time to toxin development in nitrite-treated sausages (Miller and Menichillo 1991). The observation may be related to the rapid depletion of nitrite in haem-containing products (Kim et al. 1987; Miller and Menichillo 1991). Kim *et al.* (1987) indicated that total reducing capacity of the meat system was the major factor affecting nitrite depletion. Factors affecting reducing capacity include: ascorbate, spores, iron-containing compounds and naturally-occurring meat reductants. In addition, increasing of iron levels was associated with higher pH levels. Roberts *et al.* (1981) demonstrated that higher pH was associated with toxin production.

There is a general inverse relationship between iron addition and time to toxin development, which is consistent with previous data (Miller and Menichillo 1991). As little as a doubling of iron levels above that in control samples (15–20 μg iron g^{-1}) decreases germination and toxigenesis time in *Cl. botulinum*. The time to neurotoxin detection for the acetone-treated samples, however, does not appear to follow the general trend and a mechanistic explanation for this observation is unapparent. Results may reflect a variety of situations. For example, both acetone and peroxide treatment would oxidize residual haeme iron to the ferric form, which is less available for bacterial incorporation. Alternatively, weekly bioassay testing may have been too infrequent to accurately discriminate between treatments which became toxigenic between samplings.

A significant finding of this study was that plasma appears to delay toxigenesis. The mechanism by which plasma delays toxin production remains unclear. Plasma may contain lactoferrin or transferrin, which has direct bacteriostatic activity (Payne *et al.* 1990). Furthermore, nitrite reacts with these proteins to form inhibitors to *Bacillus cereus* spore outgrowth (Custer and Hansen 1983), and may do so with *Cl. botulinum*.

These data show the importance of iron levels as a key modulator for *Cl. botulinum* outgrowth and toxigenesis. Total iron levels also influenced pH values of the model sausages. Furthermore, the study supports the hypothesis that use of blood fractions containing iron may compromise the antibotulinal efficacy of sodium nitrite in cured meat. Finally, the study demonstrates that botulinal risk from decolourized RBC fractions is generally proportional to total iron levels in the product.

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